The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression

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We have described and cloned previously a factor (MTF-1) that binds specifically to heavy metal-responsive DNA sequence elements in the enhancer/promoter region of metallothionein genes. MTF-1 is a protein of 72.5 kDa that contains six zinc fingers and multiple domains for transcriptional activation. Here we report the disruption of both alleles of the MTF-1 gene in mouse embryonic stem cells by homologous recombination. The resulting null mutant cell line fails to produce detectable amounts of MTF-1. Moreover, due to the loss of MTF-1, the endogenous metallothionein I and II genes are silent, indicating that MTF-1 is required for both their basal and zinc-induced transcription. In addition to zinc, other heavy metals, including cadmium, copper, nickel and lead, also fail to activate metal-responsive promoters in null mutant cells. However, cotransfection of an MTF-1 expression vector and metal-responsive reporter genes yields strong basal transcription that can be further boosted by zinc treatment of cells. These results demonstrate that MTF-1 is essential for metallothionein gene regulation. Finally, we present evidence that MTF-1 itself is a zinc sensor, which exhibits increased DNA binding activity upon zinc treatment.

Key words: metallothionein gene expression/targeted gene disruption/transcription factor MTF-1/zinc finger protein

Introduction

Mammalian metallothioneins are small proteins with clusters of cysteines that have a high affinity for heavy metals, in particular zinc, cadmium and copper. Metallothionein gene expression is induced by a great number of stimuli, most notably by adverse conditions such as heavy metal load, viral infection and UV- and X-irradiation [reviewed in Kaegi (1991) and Andrews (1990)]. Consequently, these proteins have been implicated in heavy metal detoxification, metal homeostasis and radical scavenging. Metal detoxification has been unambiguously demonstrated, because elimination of metallothioneins I and II by targeted gene disruption results in mice that are particularly sensitive to cadmium (Michalska and Choo, 1993; Masters et al., 1994). In addition to the ubiquitously expressed metallothionein genes I and II, there are two additional metallothioneins, III and IV, whose expression is restricted to brain and squamous epithelial tissues, respectively (Uchida et al., 1991; R.D.Palmiter, personal communication). Palmiter and colleagues originally noted the presence of conserved DNA sequence motifs in the promoters of a number of metallothionein genes, so-called metal-responsive elements (MREs), which can confer heavy metal inducibility to heterologous reporter genes (Stuart et al., 1984, 1985). We have inserted metallothionein upstream sequences into the SV40 genome, thus creating metal-dependent viruses, and also found that these upstream sequences could act over large distances as metal-responsive transcription enhancers (Serfling et al., 1985). Several groups, including ours, have described proteins that bind to metal-responsive DNA elements (Mueller et al., 1988; Séguin and Prévost, 1988; Westin and Schaffner, 1988a; Andersen et al., 1990; Searle, 1990). The candidate transcription factor (MTF-1) that specifically binds to the metal-responsive elements of metallothionein I promoters (Westin and Schaffner, 1988a) was subsequently cloned (Radtke et al., 1993). Deletion analysis of the MTF-1 cDNA identified a zinc finger-type (C_2H_2) DNA binding domain and transcriptional activation domains. When expressed in transfected mammalian cells, MTF-1 could activate transcription from metal-responsive promoters. However, activation was mostly constitutive, i.e. basal level rather than zinc-induced transcription was increased. These results could be explained by the assumption that the endogenous metal-response system was already saturated and unable to respond to additional MTF-1. However, despite the fact that no other MRE binding factor was isolated, we could not conclude with certainty that MTF-1 was involved in heavy metal-induced transcription.

In order to test definitively the role of MTF-1 in metallothionein gene regulation we have constructed an embryonic stem (ES) cell strain that has lost MTF-1 expression by means of targeted gene disruption. Using this cell line we obtained conclusive evidence that MTF-1 is required not only for heavy metal induction, but also for basal transcription of metallothionein I and II genes. We find no evidence for additional members of an MTF-1-related factor family, nor for a backup system of metal regulation by other factors in these ES cells. MTF-1 is not only essential for induction by zinc, but also mediates the response to other heavy metals such as cadmium, copper, nickel and lead.

Finally, we also present data showing that the DNA binding properties of MTF-1 are modulated by zinc treatment, indicating that this factor can respond to changes in intracellular zinc concentration.

Results

For targeted disruption of both alleles of the MTF-1 gene, we constructed two targeting vectors where most of the first zinc finger exon was replaced by a selection marker gene
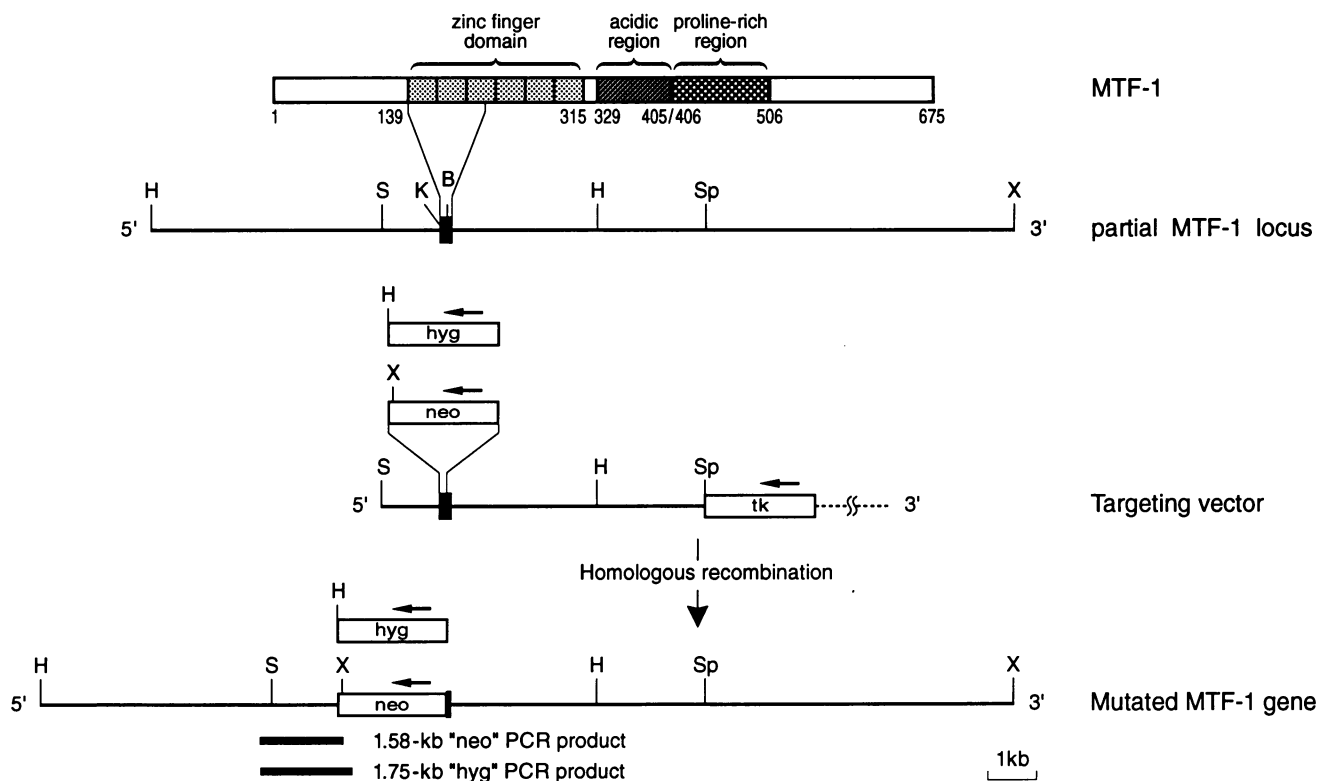
Fig. 1. Targeting vectors used to disrupt the mouse MTF-1 locus. A schematic diagram of the MTF-1 protein is shown together with the part of the MTF-1 gene locus containing the first zinc finger exon of 238 bp. The targeting vector includes 6.7 kb of genomic sequence. Two-thirds of the first zinc finger exon were replaced by either the neomycin (*neo*) or hygromycin (*hyg*) gene via homologous recombination (see Materials and methods). B, *Bam*HI; H, *Hind*III; K, *Kpn*I; S, *Sac*I; Sp, *Spe*I (not all sites shown); X, *Xba*I; thin horizontal lines, intron sequences; black rectangle, first zinc finger exon; dotted line, pBluescript.

in reverse orientation. This exon was chosen because it would eliminate DNA binding in case residual protein was produced. In addition, as was found later, the strong PGK promoter of the selection marker gene, transcribed in reverse orientation, apparently interferes with MTF-1 gene transcription with the effect that no transcripts were observed any more in an RNA mapping analysis (data not shown). The neomycin phosphotransferase and hygromycin genes used for disruption of the first and second allele, respectively, yielded homologous insert colonies at frequencies of 1/8 and 1/16, respectively (Figure 1; see also Materials and methods). ES cells were electroporated consecutively with these two vectors and homozygous mutant (−/−) ES cell colonies were identified by PCR and confirmed by Southern blot analysis (data not shown).

To examine whether the −/− ES cells obtained by this procedure still contained any residual MTF-1-like binding activity, perhaps due to a related and/or redundant protein, we performed gel retardation analyses (Figure 2). Nuclear extracts from control +/+ ES cells (wild-type for the MTF-1 locus but G418-resistant due to non-homologous vector integration) and −/− ES cells had indistinguishable amounts of the ubiquitous transcription factor Sp1 (Figure 2, lanes 1−4). However, only the +/+ ES cell contained any MRE binding activity (Figure 2, lanes 5−8). MTF-1 binding activity was increased several-fold by zinc treatment of the cells, while the signal intensity of Sp1 remained unchanged (Figure 2, lanes 1 and 2 versus 5 and 6; this effect will be addressed in more detail below). The −/− ES cells showed no detectable MTF-1, either before or after zinc treatment

(Figure 2, lanes 7 and 8). This result confirms the successful disruption of the MTF-1 locus and also indicates that there are no additional proteins binding to the MRE-site oligonucleotides tested.

To investigate the role of MTF-1 in metallothionein gene regulation, mRNA levels of the endogenous metallothionein I and II genes were measured. As shown in Figure 3, the mRNA level of MTF-1 itself is only marginally affected by heavy metal treatment, essentially ruling out any transcriptional autoregulation. As expected, levels of metallothionein I and II gene transcripts were greatly increased upon treatment with zinc, cadmium or other heavy metals, like copper, nickel and lead (Figure 3, lane 1 versus lanes 2−6). In marked contrast, both before and after treatment of the cells with zinc or other heavy metals, the −/− ES cells contained undetectable amounts of MTF-1 transcripts and little, if any, metallothionein mRNA (<1% of basal level in wild-type cells) (Figure 3, lanes 7−12).

Next we sought to restore metallothionein transcription by introducing the MTF-1 cDNA into null mutant ES cells in transient transfection experiments. Wild-type and null mutant cells were transfected with reporter genes under the control of either four copies of the strong metal-responsive element MREd (4×MREd OVEC), or the complete mouse metallothionein I promoter (MT-I OVEC). Treatment of +/+ ES cells with 400 μM zinc resulted in a 10-fold increase of transcription from a transfected 4×MREd reporter gene (Figure 4A, lanes 1 and 2). Cotransfection of 2.5 μg MTF-1 expression vector dramatically increased both basal and zinc-induced transcription (Figure 4A, lanes 3 and 4). By contrast,
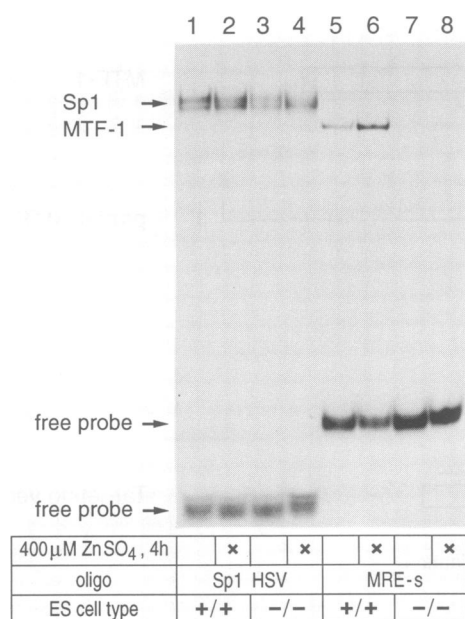
Fig. 2. Gel retardation analysis using nuclear extracts from MTF-1 +/+ ES cells or MTF-1 −/− ES cells and end-labeled oligonucleotides. For zinc induction, ES cells were treated with 400 μM ZnSO$_4$ or 80 μM CdCl$_2$ in growth medium (DMEM, 20% FCS, 0.1 mM 2-mercaptoethanol) for 4 h before harvesting, as indicated. Sp1 was used as an internal control for total protein in the reaction mixture because it is not influenced by zinc treatment of cells (Radtke *et al.*, 1993). Electrophoretic mobility shift (bandshift) assays were performed with oligonucleotides containing Sp1 and MTF-1 binding sites (HSV Sp1 and MRE-s, respectively), as described in Radtke *et al.* (1993). The positions of the respective protein–DNA complexes are indicated.

in the −/− ES cell line, reporter gene expression was barely detectable either with or without zinc treatment (Figure 4A, lanes 5 and 6). Only after cotransfection of the cloned MTF-1 gene was transcription restored to the levels observed with MTF-1-cotransfected +/+ ES cells (Figure 4A, lanes 3 and 4, and 7 and 8). Similar results were obtained using a reporter gene driven by the complete metallothionein I promoter instead of the 4×MREd promoter; increasing concentrations of MTF-1 resulted in increased levels of both uninduced and induced transcription (Figure 4B and C).

Finally, we have addressed the question whether zinc-induced DNA binding activity of MTF-1 is due to the same, or to different, mechanisms when observed *in vitro* versus *in vivo*. Figure 5 shows that in nuclear extracts which are not supplemented with zinc, MTF-1 is incompletely zinc-saturated, and that binding activity can be greatly increased by zinc addition. This effect is much more pronounced than with Sp1, another mammalian zinc finger factor, indicating that MTF-1 requires a higher zinc concentration for optimal DNA binding than does Sp1.

We had also noted that after metal treatment of cells prior to extract preparation, there is always a several-fold increase in MTF-1 bandshift activity in nuclear extracts (Figure 5A versus B). It was unclear whether this was due to a higher starting concentration of zinc ions in the extracts, or to a separate mechanism of some kind. Here we show that the latter is the case. Increasing amounts of zinc added to either the extracts of untreated or zinc-pretreated cells do not level out the several-fold difference between them, even at zinc
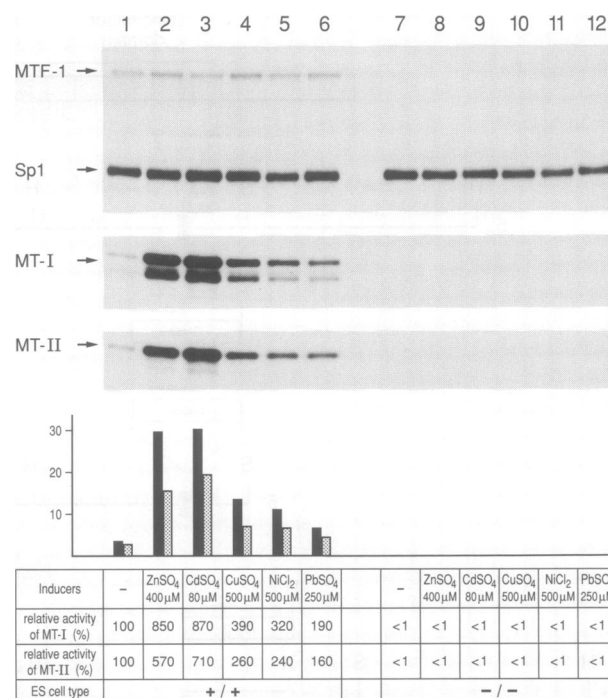

Fig. 3. Loss of metallothionein gene regulation in MTF-1 −/− embryonic stem cells. Transcript levels of MTF-1, metallothionein I and metallothionein II were determined in MTF-1 +/+ and MTF-1 −/− ES cells. Each lane represents protected RNA segments obtained after hybridizing 20 μg of cytoplasmic RNA with MTF-1, Sp1, metallothionein I and metallothionein II antisense RNA probes simultaneously. Sp1 transcript levels were used as internal controls because they are not affected by heavy metal treatment. Before harvesting, cells were treated with different metal salts, as indicated. In the presence of MTF-1, a several-fold induction of both metallothionein genes I and II can be observed upon challenging +/+ ES cells with different heavy metals (lanes 1−6). The transcript levels of Sp1 and MTF-1 itself are at most marginally influenced by this treatment. However, neither basal nor heavy metal-induced transcripts of metallothionein genes can be detected in −/− ES cells which lack MTF-1 (lanes 1−7). The residual metallothionein I and II levels in −/− ES were determined by PhosphorImager analysis to be <1% of the basal level observed in +/+ ES cells (lane 1 versus lanes 7−12).

concentrations that largely exceed the conditions of cell treatment (Figure 5). Thus we have identified two separate ways to yield higher concentrations of active MTF-1.

Firstly, the protein is suboptimally saturated with zinc *in vitro*, and upon zinc addition an increasing fraction can be converted to a DNA binding form. From this it appears likely that the zinc fingers of MTF-1 are, directly or indirectly, involved in metal induction (see Discussion).

Secondly, there is another mechanism in zinc-treated cells that mobilizes a several-fold elevated DNA binding activity, perhaps as a result of *de novo* protein synthesis, or by the modification/release of a pre-existing MTF-1 precursor.

Discussion

MTF-1 gene disruption

We have identified and cloned previously a transcription factor that selectively binds to the MREs of metallothionein promoters (Westin and Schaffner, 1988a; Radtke *et al.*, 1993). When transfected into mammalian cells, MTF-1 strongly stimulated expression of MRE-containing promoters. However, activation was largely constitutive, and
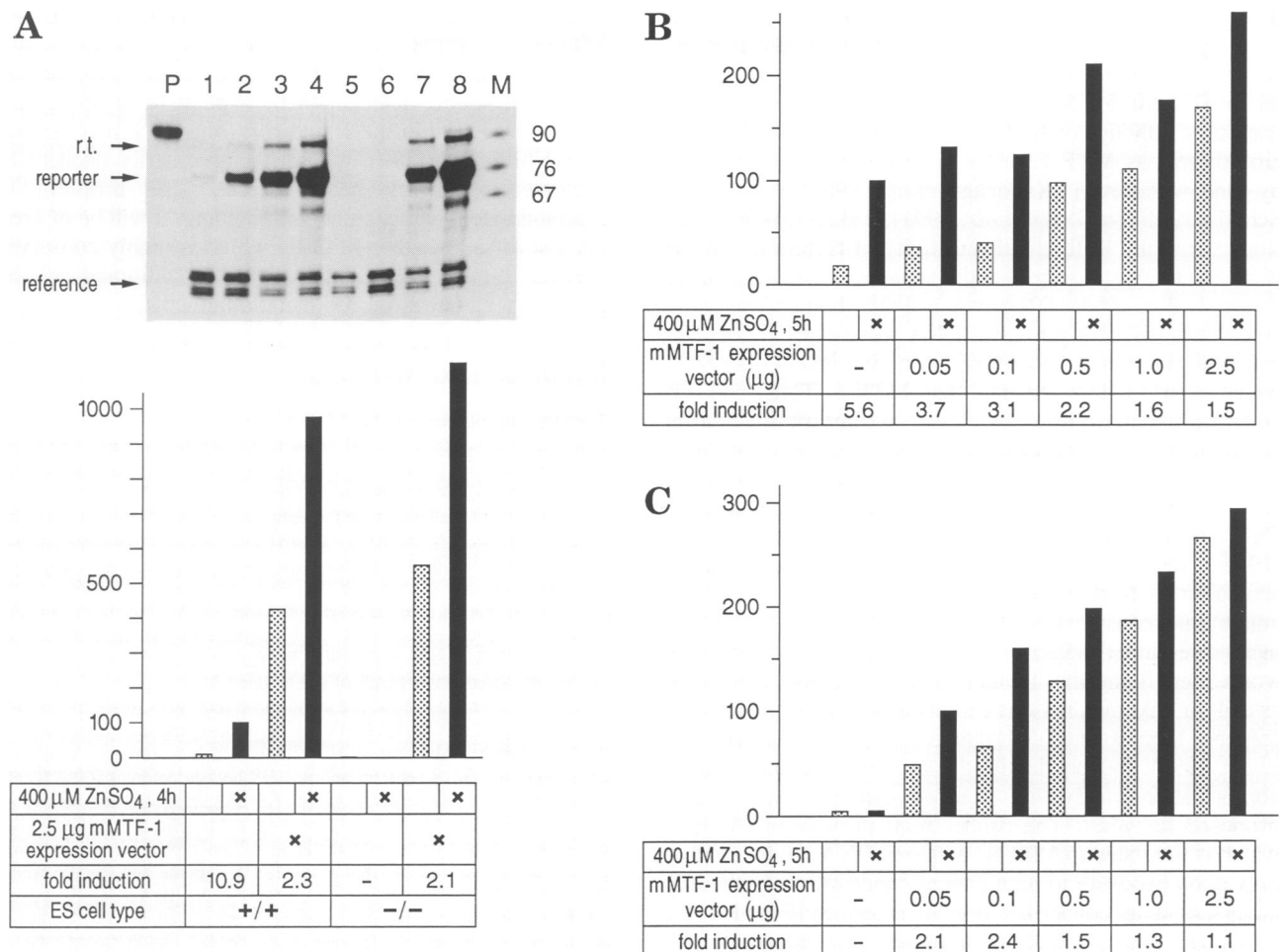
A

400 µM ZnSO$_4$, 4h		×		×		×		×
2.5 µg mMTF-1 expression vector			×	×			×	×
fold induction		10.9	2.3			–		2.1
ES cell type		+/+				–/–		

B

400 µM ZnSO$_4$, 5h		×		×		×		×		×		×
mMTF-1 expression vector (µg)	–		0.05		0.1		0.5		1.0		2.5	
fold induction	5.6		3.7		3.1		2.2		1.6		1.5	

C

400 µM ZnSO$_4$, 5h		×		×		×		×		×		×
mMTF-1 expression vector (µg)	–		0.05		0.1		0.5		1.0		2.5	
fold induction	–		2.1		2.4		1.5		1.3		1.1	

Fig. 4. S1 nuclease mapping of ES cells. ES cells were treated before harvesting as described in Figure 2. 400 µM ZnSO$_4$ were added to the medium before harvesting of cells, as indicated. (A) 20 µg 4×MREd OVEC reporter construct transfected either with or without MTF-1 expression vector. (B) 20 µg MT-I OVEC reporter construct transfected with increasing amounts of MTF-1 expression vector into +/+ ES cells or (C) into –/– ES cells. OVEC-ref (0.5 µg) was cotransfected as an internal standard for transfection efficiency.

some queries remained about the role of MTF-1 in metal induction. To settle this question, we have eliminated MTF-1 expression by means of targeted gene disruption in embryonic stem cells (ES cells) of the mouse.

Our results show that both basal and metal-inducible transcription from natural and synthetic metal-responsive promoters is lost upon disruption of the MTF-1 gene and can be restored to a large extent by cotransfection of the MTF-1 expression vector. The fact that no bandshift activity was detectable using extracts from null mutant cells indicates that no other factors with related DNA binding properties exist in these cells. From this we conclude that MTF-1 is the crucial transcription factor for metallothionein gene induction. In addition, MTF-1 is also absolutely required for basal level transcription of metallothionein genes I and II.

This is surprising, because *in vivo* footprinting data of Barbara Wold and her colleagues have shown that binding sites for constitutive factors Sp1 and USF in the metallothionein I gene promoter are fully occupied, even in the absence of heavy metals (Mueller *et al.*, 1988). These factors apparently cannot support basal transcription; they may nevertheless have an auxiliary role in concert with MTF-1. The finding that MTF-1 is also necessary for the basal transcription of metallothionein genes implies a role
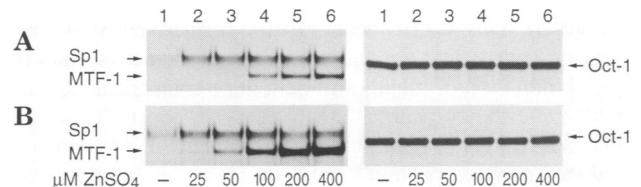

Fig. 5. Zinc-induced DNA binding of MTF-1 from untreated and zinc-treated cells. Nuclear extracts were prepared from untreated mouse 3T6 cells (A) and cells treated with 100 µM zinc sulfate for 4 h prior to harvest and nuclear extract preparation (B). Lanes 1–6, increasing amounts of zinc ions were added to the binding buffer before the DNA–protein binding reaction and gel electrophoresis. The left panel shows bandshifts with the MREd oligonucleotide, which binds both to Sp1 and MTF-1. The right panel shows control bandshifts with an 'octamer' site oligonucleotide and Oct-1 factor, whose DNA binding domain does not contain zinc fingers and is known to be unaffected by zinc concentration (Westin and Schaffner, 1988b).

for MTF-1 (and metallothioneins) under non-stressed, physiological conditions.

Zinc-induced DNA binding of MTF-1

We have observed an increase in DNA binding of MTF-1 in response to either zinc treatment of extracts or zinc

treatment of living cells prior to extract preparation. Upon investigation we found that MTF-1 factor *in vitro* is more sensitive to the environmental zinc concentration than Sp1 factor (Figure 5). Sp1 does not mediate a heavy metal response, although it harbors the same type of His$_2$Cys$_2$ zinc fingers as MTF-1, and can be reversibly inactivated by zinc withdrawal (Kadonaga *et al.*, 1987; Westin and Schaffner, 1988b; Zeng *et al.*, 1991). Taken together, our studies reveal a differential sensitivity of factors to zinc *in vitro*, which may also reflect the situation *in vivo*. Octamer factor 1 that binds DNA via a POU homeodomain is completely insensitive to zinc. RNA polymerase II contains essential zinc but is less sensitive to zinc levels than Sp1, which in turn is less sensitive than MTF-1. Thus, one can envisage a scenario whereby classes of transcription factors are regulated by zinc availability (Westin and Schaffner, 1988a,b; Zeng *et al.*, 1991). MTF-1 would only bind zinc, and thus become DNA binding-competent, at high zinc load. This model, proposed originally by Westin and Schaffner (1988a), does not take into account the largely constitutive activation of transcription by transfected mouse MTF-1, unless one considers a saturation effect due to MTF-1 overexpression (Radtke *et al.*, 1993; in this context it is worth mentioning that human MTF-1 was also cloned by us and for unknown reasons confers a more pronounced zinc response in transfected cells than the mouse factor; Brugnera *et al.*, 1994). Independently, this model of a metal-induced conformational activation of a regulator protein was also proposed for yeast: The copper regulatory factor ACE-1, which is unrelated to MTF-1, acquires DNA binding ability only upon exposure to high copper concentration, whereby a DNA binding 'copper fist' domain is formed [Fuerst *et al.*, 1988; Hu *et al.*, 1990; for a review see Heuchel *et al.* (1994)].

We have also discovered an independent mechanism that leads to elevated MTF-1 binding activity. Extracts from cells pretreated with zinc always contain several-fold more DNA binding MTF-1 than control extracts, irrespective of the amount of zinc added to the binding reaction (Figure 5A versus B). This MTF-1 accumulation is relatively slow, over several hours (data not shown). Thus, it cannot explain the rapid induction of metallothionein gene transcription, but it may nevertheless contribute a delayed boost. This slow increase in MTF-1 binding activity is presumably due to zinc-induced *de novo* protein synthesis. However, a conversion of some inactive or less active precursor form of MTF-1 to a more active or higher affinity one cannot be excluded. In any case, transcriptional activation of the MTF-1 gene itself is ruled out, because MTF-1 mRNA is barely affected by metal treatment of cells (Figure 3, lane 1 versus lanes 2−6). Recently Palmiter (1994) has also reported that MTF-1 induced constitutive transcription upon stable cell transformation, and he obtained evidence for a negative regulatory mechanism. He proposed that MTF-1 is a constitutive factor controlled by a zinc-sensitive inhibitor, tentatively designated MTI, which would be titrated out in the transformation experiments. It was thus suggested that the key to understanding metal regulation would be the identification of such an inhibitor and the determination of how it interacts with MTF-1 and responds to metals. Our data presented previously (Radtke *et al.*, 1993) and here are also compatible with negative regulation by an inhibitor that controls MTF-1 availability. However, we believe that the

direct influence of zinc on the DNA binding properties of MTF-1 *in vitro* strongly suggests that MTF-1 is itself a zinc sensor, or at least part of the *in vivo* sensing mechanism, rather than being acted upon by a putative metal-sensing mechanism. Within the framework of such a mechanism, an inhibitor of MTF-1 would not necessarily need to be regulated itself by heavy metal. The problem of metallothionein induction notwithstanding, it will be of great interest to see whether MTF-1, which is highly conserved between human and mouse, regulates additional cellular genes other than those for metallothioneins.

Materials and methods

Targeted disruption of the MTF-1 gene
Using the 520 bp *Kpn*I−*Bsp*HI probe harboring the complete zinc finger region of the mMTF-1 cDNA, a 17 kb DNA fragment was isolated from a genomic (AB-1; McMahon and Bradley, 1990) λ GEM-11 library (kind gift from Dr U.Mueller). In the targeting vector, based on pBluescript KS (Stratagene), the 146 bp *Kpn*I−*Bam*HI zinc finger exon fragment was replaced by either a blunt-ended neomycin phosphotransferase or hygromycin expression cassette (Soriano *et al.*, 1991; Ruffner *et al.*, 1993) in the antisense orientation. Into the polylinker *Eco*RI site 5' of the neo expression cassette, the blunt-ended *Nco*I−*Sal*I fragment of the upstream mouse sequence (UMS; Heard *et al.*, 1987; Ruffner *et al.*, 1993) was inserted to reduce unwanted readthrough of the targeted MTF-1 gene. 5' and 3' of the mutated exon are 1.2 and 5.5 kb of intron sequences, respectively. The 3' intron region is followed by the herpesvirus thymidine kinase gene for double selection (Mansour *et al.*, 1988), which resulted in a 4.5-fold enrichment for G418-resistant and tk⁻ GS-ES cell colonies (GS-ES cells are derived from mouse strain Agouti 129/SV) after selection with G418 (0.4 mg/ml) and FIAU (0.2 μM) for 9 days. Identification of GS-ES cell clones with homologous recombination of the replacement vector was performed as described (Soriano *et al.*, 1991), with the first specific primer (GEN131) 5'-TGTGTATGTCTTCTTGGGGATGGAACC-3' corresponding to genomic sequence upstream of the targeting vector sequence and the second primer (P6; Ruffner *et al.*, 1993) 5'-ATTCGCAGCGCA-TCGCCTTCTATCGCC-3' corresponding to the 3' end of the neo cassette. Cycling was performed with an initial denaturation at 95°C for 3 min, then 38 cycles at 94°C for 0.5 min, 63°C for 0.5 min, 65°C for 2 min and final extension at 65°C for 10 min. By Southern blot analyses, all PCR-positive clones were found to be heterozygous for the MTF-1 locus, without additional integrations of the targeting vector. The heterozygous clone 34F6 was electroporated with the hygromycin targeting vector. After 8 days of selection (100 μg hygromycin B/ml and 0.2 μM FIAU/ml), resistant clones were picked and analyzed by PCR as before, with the first primer (GEN136) 5'-ATGTCTTCTTGGGGATGGAACC-3' and the second primer (P7; Ruffner *et al.*, 1993) 5'-CCGGGACTGTCGGGCGTACACA-3'. Double selection yielded 5-fold enrichment, and one ES cell clone out of 16 was correctly targeted. As before, PCR-positive clones were verified by genomic Southern blot analysis.

Culturing conditions for +/+ and −/− ES cells
ES cells were grown on irradiated SNL76/7 feeder cells (Soriano *et al.*, 1991) without antibiotics. 24 h before harvesting, cells were trypsinized and depleted of feeder cells by two passages for 1 h each on non-gelatinized cell culture dishes. This method ensures a >90% depletion of feeder cells. The remaining ES cells were plated on gelatine (1%) pretreated cell culture dishes for ~12 h. For heavy metal induction, ES cells were treated with 400 μM ZnSO₄ or 80 μM CdCl₂ in growth medium (DMEM, 20% FCS, 0.1 mM 2-mercaptoethanol), as indicated. Note that the 400 μM ZnSO₄ or 80 μM CdCl₂ used for induction are higher than the usual concentrations for metal induction of cultured cell lines. However, substantial amounts of heavy metal are probably bound to the highly concentrated serum proteins of ES cell medium.

Reporter DNAs, transfections, transcript mappings and gel retardation assays
Metal-responsive OVEC reporter genes, transfections and S1 nuclease mapping of transcripts were performed as described previously (Radtke *et al.*, 1993).

Mapping of transcripts from MTF-1, Sp1 and metallothionein I and II genes. Preparation of cytoplasmic RNA was according to Radtke *et al.* (1993).

RNase protection was performed as described in Mitchell *et al.* (1991). MTF-1 antisense probe: full-length, 355 nt; 287 nt protected, corresponding to positions +373 (*Sna*BI) to +660 (*Bam*HI). Metallothionein I antisense probe: full-length, 259 nt; 105 nt protected, corresponding to positions +1 to +105. Metallothionein II antisense probe: full-length, 274 nt; 67 nt protected, corresponding to positions +340 to +407 relative to the transcription start site (Searle *et al.*, 1984). Sp1 antisense probe is described in Mitchell *et al.* (1991). The signal intensities of protected RNA fragments were quantified using a PhosphorImager (Molecular Dynamics) and data were normalized against Sp1 transcript signals including the specific activity of the individual probes. This sensitive detection method allowed us to determine that in MTF-1 −/− ES cells, transcript levels of metallothionein I and II genes were <1% of the basal level in MTF-1 +/+ cells. The autoradiograph from one experiment was cut into strips and the bands arranged for easy reference.

Preparation of nuclear extracts and gel retardation assays, including the oligonucleotides used, are described in Radtke *et al.* (1993).

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